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MORPHO-MOLECULAR CHARACTERIZATION OF *TRICHODERMA* ISOLATES FROM RHIZOSPHERIC SOILS OF VEGETABLES IN PAKISTAN

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ABSTRACT

Trichoderma, a major fungal genus attaining importance due to its diverse application in biological control programs and is considered a substitute for chemical pesticides. This research was conducted to characterize various *Trichoderma* species isolated from rhizospheric soil samples morphologically followed by its confirmation using molecular tools. A systematic survey of *Trichoderma* populations associated with soils of different vegetable hosts would enable a clear picture of the distribution of species in the region. Samples were collected from the rhizospheres of a variety of vegetable hosts and obtained numerous *Trichoderma* isolates (*T. harzianum, T. viride, T. hamatum, T. longibrachiatum, T. asperellum, T. koningii* and *T. longipile*). Morphological characteristics revealed that *T. harzianum* resembles *T. viride* but is more pigmented with confined rings than *T. viride* and other associated species. *T. viride* sporulation was more rapid than other species, producing a soft mat on PDA media. *T. viride* produces a sweet smell of coconut; *T. asperellum* produces a misty odour while *T. longibrachiatum* produces a yellow pigmentation in the media. Fifty out of 200 morphologically identified species were genetically characterized using universal primers (ITS-1 and ITS-4). ITS-based sequencing resulted in a product of 650 bp in all the isolates. The sequencing of these isolates showed five different species. As per rDNA, the species identified are: *T. harzianum, T. hamatum, T. longibrachiatum, T. asperellum* and *T. viride* with 98-100% sequence similarities to other related *Trichoderma* isolates reported from China, India, Mexico, USA, Portugal, Germany, Spain and Brazil. Bioinformatics analysis was conducted using maximum parsimony (MP) that supports the resemblance of the present study *Trichoderma* species with species reported from other countries. It is concluded that *Trichoderma* strains with biocontrol activity are genetically different compared to the pathogenic ones. The findings of this study help in providing an opportunity to test these isolates against different plant pathogens and ultimately leads to the development of bio-pesticides that could be eco-friendly and cost-effective with no chance of resistance development.

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INTRODUCTION

Trichoderma belongs to the class Deuteromycetes and members of this genus are usually present in all types of soils (Faizova and Perepelkina, 2015). It is a soil-borne filamentous fungus which is capable of parasitizing several plant pathogenic fungi by producing sexual and asexual spores and is widely distributed in the soil, plant material, decaying vegetation and wood. *Trichoderma* spp. a biocontrol agent directly competes for nutrients and space and indirectly behaves as mycoparasitic fungi against phytopathogens (Jeger *et al.*, 2009; Faraz *et al.*, 2022). *Trichoderma* is an important source to produce enzymes and is very beneficial in the recycling of waste material. It gains more interest as a fungal antagonist for soil-borne and foliar pathogens (Srivastava *et al.*, 2015; Mukhopadhyay and Kumar, 2020; Banday *et al.*, 2022). They also produce specific enzymes and antibiotics, which help in controlling the growth of other pathogens and boosting crop yield (Sood *et al.*, 2020).

Trichoderma appears as a promising biocontrol agent and plays a significant role in the management of plant diseases. Due to its ability to regulate pathogen populations, improve vegetative growth and protect plants under varied agricultural situations, it has broad-spectrum efficacy. Additionally, it is utilized as a soil supplement or inoculant to enhance the fertility, decomposition, and biodegradation of the soil. As bio-pesticides, bio-fertilizers, and growth regulators, these are offered for sale. Additionally, it increases plant height and productivity in a variety of environments, including nurseries, greenhouses, and fields used for the cultivation of ornamental plants and fruit trees (Kumar and Gupta, 1999). *Trichoderma* fungi are typically found on various substrates including decaying wood (Samuels, 1996). Some of them like *Trichoderma ressei* is an economically significant producer of industrial enzymes (Kubicek and Penttila, 1998), antibiotics (Sivasithamparam and Ghisalberti, 1998), and have also been used as biocontrol agents (i.e., *T. harzianum*, *T. atroviride* and *T. asperellum*) against plant pathogens (Harman, 2000).

Trichoderma is easily identified on culture media, which produces a large number of characteristics mycelial mat, growth on the media, small, green or white conidia, and phialides present on the profusely or meagerlybranched conidiophores. The intricacy and closely comparable features of the species make it challenging and confusing to identify isolates down to the species level. However, there is still considerable interest in finding more efficient mycoparasitic fungi, especially within *Trichoderma* spp., which differ considerably in their biocontrol effectiveness. The differentiating characteristic of the *Trichoderma* isolates includes mycelial growth rate and colony appearance, as well as microscopic morphological traits including phialides and phialospores (Seaby, 1996). These can also be distinguished by sequence analysis of ribosomal DNA (Castle *et al.*, 1998) (Muthumeenakshi *et al.*, 1994; Muthumeenakshi and Mills, 1995; Ospina-Giraldo *et al.*, 1999). Molecular characterization of the potential biocontrol agents using Internal Transcribed Spacer-Polymerase Chain Reaction (ITS-PCR), helps to determine the diversity and identification. Molecular analysis of several strains revealed that classification based on morphological data has been erroneous to a great extent resulting in the re-classification of several isolates and species (Hatvani *et al.*, 2019). The classical approach for morphological characterization with some specific characteristics was reliable for most fungi up to its species level. Morphological data for *Trichoderma* is more prone to variability, and error while roughly 30-50% of identified characteristics were erroneously identified (Kubicek *et al.*, 2003). Thus, the present study was performed to characterize the cryptic species of *Trichoderma* associated with vegetable crops grown in different districts of Pakistan using morphological and molecular approaches.

MATERIALS AND METHODS

Isolation and Morphological Identification

Soil samples (n = 200) were collected from the root zone of different vegetable hosts being grown in Rawalpindi, Chakwal, Faisalabad and Multan districts of Punjab, Pakistan. Soil samples from healthy plants were appropriately labelled and sealed in polythene bags before being delivered to the Biotechnology Lab at CABI Rawalpindi, Pakistan. The collected samples were placed in a refrigerator at 4°C.

The multi-dilution method was used to isolate the *Trichoderma* species on potato dextrose agar (PDA) media (Rahman *et al.*, 2011). Fungal colonies were collected by using the single spore method and then transferred onto the fresh PDA plates and incubated at 28 ± 2°C for 96 h (Samson *et al.*, 2010). The purified isolates were preserved at 4°C and further used during

the study. The morphological and cultural characteristics of *Trichoderma* isolates were analyzed under the microscope as reported earlier (Samuels *et al.*, 2002). Mycelial discs (6 mm) of actively growing culture of respective isolates of *Trichoderma* were inoculated in the periphery of the PDA plates and incubated at 28 ± 2°C for one week. Colony radius was measured at 24, 48 and 72 h. The fungal growth rate experiment was repeated in triplicate and the results were averaged for each isolate. Morphological characteristics (conidia, conidiophore, phialides, phialide length) were observed by using a compound microscope at 40-100X magnification. Additional characteristics were also observed on PDA plates including the presence of pigments, green conidia, odour, mycelium pattern, growth and colony appearance.

Molecular Characterization of *Trichoderma* Isolates

The cultures of *Trichoderma* isolates were maintained on PDA broth at 25°C for 3 days. Mycelial mat was collected on filter paper, washed with distilled water 2–3 times, frozen and used for DNA extraction. For a detailed and precise identification of the *Trichoderma* isolates, the genomic DNA was extracted from the monosporic culture by using the DNA isolation kit: Qiagen (Sambrook *et al.*, 1989). DNA was resuspended in 50 µL TE buffer and run on gel electrophoresis to confirm the desired length of bands. The fungal universal internal transcribed spacer (ITS) region ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) were used described by White *et al.* (1990) and used to amplify the fragment of *Trichoderma* species. PCR amplifications were performed by using the thermocycler (Bio-Rad) with an initial denaturation at 94°C for 1 min followed by 36 repetitive cycles; annealed at 55°C for 1 min with extension at 72°C for 2 min and a final extension at 72°C for 10 minutes.

The PCR amplified products were analyzed by using the 1% agarose gel containing the Bio-safe DNA staining solution in the Tris-acetate EDTA (TAE) buffer. The amplified samples were compared with a 1 kb DNA ladder and purified by using the Qiagen purification kit (Chen *et al.*, 2009). PCR amplified products were again purified by using Qiagen purified Kit for obtaining a highly purified genome and then further sequenced at Florida State University, sequencing facilities in Tallahassee Florida. For further analysis, the sequences were aligned by using the Sanger sequencing method (Sanger *et al.*, 1977) and submitted to GenBank (NCBI Database).

Phylogenetic Analysis

Trichoderma gene sequences of the isolates were compared with ITS sequences available in the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov) with the help of BLAST tool. Multiple sequence alignment was performed using Clustal X (1.1). The method of Jukes and Cantor (1969) was used to analyze closely related species through evolutionary distances. Sequences were aligned and a phylogenetic dendrogram was constructed by the neighbour-joining method and tree topologies were evaluated by performing bootstrap analysis of 1000 data sets using MEGA 7 (Molecular Evolutionary Genetic Analysis) (Tamura *et al.*, 2013).

RESULTS

Surveys and Isolations

A detailed survey of vegetable growing areas of districts Chakwal, Faisalabad, Multan and Rawalpindi of Punjab Province was conducted. The surveyed areas were made up of four distinct agro-climatic environments with various types of soil and topographical identity. The soil samples were collected randomly from the healthy fields for the morphologically distinct species of *Trichoderma*. The samples were thoroughly processed for isolation and identification (Bourguignon, 2008). A total of 80% of isolated species from collected samples were *T. asperellum,* 10% *T. longibrachiatum*, 4% *T. viride*, 2% *T. hamatum,* 2% *T. harzianum*, 1% *T. longipile* and 1% *T. koningii* (Figure 1).

Morphological Characterization

Morphological identifications of the isolates were performed based on the identification keys of the genus "*Trichoderma"*. The isolated samples were examined based on their different attributes i.e., colony colour, growth, mycelium pattern, pigmentation conidia, conidiophores and phialides (Chaverri *et al.*, 2003). Careful morphological observations were used for strain and species identification. A wide diversity has been found among the collected isolates of *Trichoderma* spp. in vegetable-growing areas of the Punjab district. Using keys from "the genus *Trichoderma*", *T. harzianum* has a white to light green to dull green colour, rapid growth in concentric rings and septate, smooth mycelium and conidia were colourless and globose.

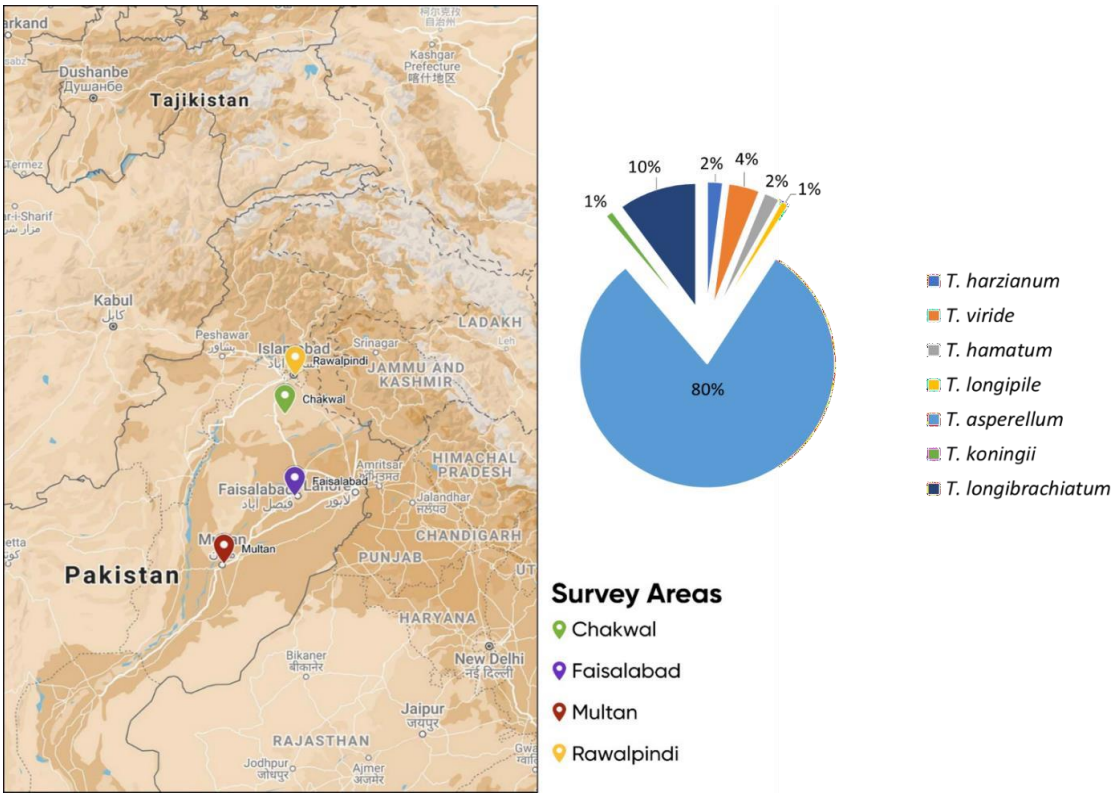

Figure 1. Sampling regions for the collection of *Trichoderma* species and their isolated frequency. The soil samples were collected based on their soil type and geographical area.

The conidiophores were highly branched and their phialides were short, pin-shaped, and narrow from the base (Figure 2A). Cultural characteristics and colony appearance were already been studied and confirmed. The colony colour of *T. viride* was light green to dark green, with fast scattered, and irregular growth. The mycelium was septate, colourless, globose and branched; whereas phialides were slender slim and small (Figure 2B). *T. hamatum* has dark green colony colour which is compact and speedily growing with no growth zones. The conidia were globose, conidiophores were branched and phialides were short and pear-shaped (Figure 2C). *T. longipile* colony showed white mycelia with compact growth having some light green portions in the middle. The fungus is slow growing with a coconut-like smell. The conidiophores were branched with short and pear-shaped phialides and were seen under a compound microscope (Figure 2D). *T. asperellum* represents the loose and compact with fast growth with loose powder-like growth. The mycelium was hyaline and highly branched with wart-like conidia, rough surface, globose and loose and compact phialides and mist-like smell of culture (Figure 2E). The light green colony colour with one concentric ring was observed in the case of *T. koningii*. The fungus was fast growing, having hyaline and branched mycelium. The conidia were rough-walled, the conidiophores were highly branched and the phialides were bottle-shaped (Figure 2F).

The fungus *T. longibrachiatum* has been divided into three sections based on their growth speed as slow growing, normal growing and fast growing. The slow-growing *T. longibrachiatum* has white mycelium which later on turns light green. The mycelium was hyaline, septate with smooth-walled conidia. The conidiophores were highly branched containing bottle-shaped phialides (Figure 2G). The isolates of *T. longibrachiatum* having normal growth did not produce any yellow colour and the mycelium was loose powder-like. The collected isolates had a pleasant smell with globose conidia, branched conidiophores and bottle-shaped phialides (Figure 2H). The fast-growing *T. longibrachiatum* has multiple concentric rings with no white growth. The mycelium was hyaline, ellipsoidal with smooth-walled branched conidia and bottle shape phialides (Figure 2I) (Table: 1).

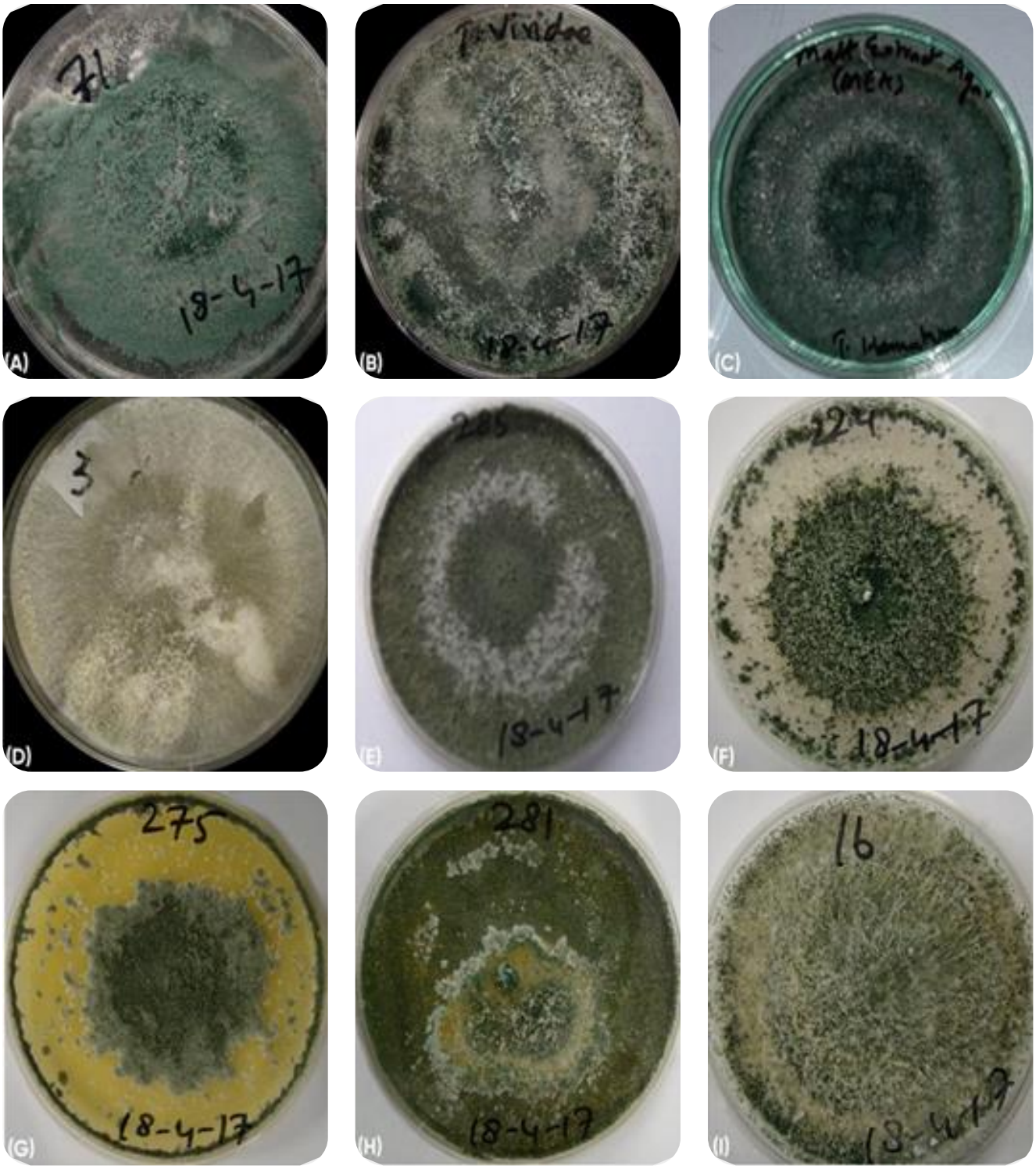

Figure 2: The growth pattern of different isolated Trichoderma fungi. **(A)** *T. harzianum* showing the white green to light green and dull green color. **(B)** *T. viride* was scattered, irregular, light green to dark green. **(C)** *T. hamatum* have dark green colony color which is compacted. **(D)** *T. longipile* have white mycelial with compact growth. **(E)** *T. asperellum* representing the loose and compact color with fast growth. **(F)** The light green colony color with one concentric ring was observed in case of *T. koningii.* **(G)** Slow growing *T. longibrachiatum* has white mycelium which later on turns into light green. **(H)** *T. longibrachiatum* having normal growth doesn't produce any yellow color and the mycelium is loose powdered like. **(I)** The fast growing *T. longibrachiatum* have multiple concentric rings.

Table 1. Morphological features of the isolated *Trichoderma* species from different areas of Punjab. The isolates were categorized based on their colony color, growth pattern, mycelium structures, odor, conidia, conidiophores and phialides (Gams & Bissett, 2002).

Fungi	Colony color	Growth	Mycelium	Odor	Conidia	Conidiophore	Phialides
T. harzianum	White green to light green and dull green	Fast growth	Septate, colorless	Normal	Globose and smooth	Highly branched	Short, pin shaped, narrow from the base
T. viride	Scattered, irregular, light green to darkgreen	Fast growth	Septate & colorless	Strong coconutlike smell	Globose	branched	Slender or slim andsmall
T. hamatum	Dark green colony, compact	Fast growthNo growth Zone		No smell	Globose	branched	Short and pear shape
T. longipile	White mycelial growth compact, somelight green in the center	Slow growth		Coconut smell	No spore as suchobserved	branched	short and pear shape
T. asperellum	Loose and compact	Fast growth	Hyaline & highly branched	-	Wart like conidia, rough surface, globose		Loose and compact
T. koningii	One concentric ring from the center of inoculum, light green	Fast growth	Hyaline & branched	Oblong like	Rough walled	Highly branched	Bottle shape
T. longibrachiatum	White mycelium laterally turns to light green pigmentation in the media,compact	Slow growth	Hyaline & septate	No smell	Smooth walled conidia	Highly branched	Bottle shape
T. longibrachiatum	No yellow color produces, loose as powdered form	Normal growth		Pleasant smell	Globose spore	branched	Bottle shaped
T. longibrachiatum	Multiple concentric rings with no whitegrowth	Fast growth	Hyaline	Ellipsoidal	Smooth walled conidia	Highly branched	Bottle shape

Molecular Characterization

Morphological identification was not enough to identify species, therefore, sequence analysis of fifty isolates was performed to confirm species identity, which initially has been done based solely on morphological parameters. A comparison of oligonucleotide fragments of rDNA sequences, with reference sequences from public databases, showed that they were very similar. (Figure 3).

Morphologically characterized isolates (*T.*Purified samples were sequenced and were found *asperellum, T. viride, T. harzianum, T. hamatum, T.*identified using BLAST tool in the NCBI to determine *longibrachiatum*) were amplified by using thethe homogeneity with already reported sequences universal primers ITS1-ITS4 regions (Mazrou *et*available in the database. The bioinformatic analysis *al.*, 2020). An amplified product of 650 bp waswas performed to find out the evolutionary relationship observed in all the amplified isolates whichof *Trichoderma* species with already reported ones. confirmed their presence on gel electrophoresis

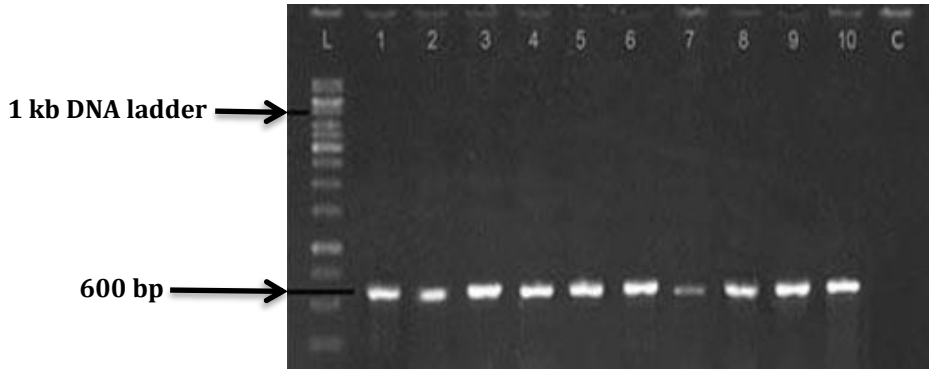

Figure 3. Two isolates from each *Trichoderma* group *(T. asperellum, T. viride, T. harzianum, T. hamatum, T. longibrachiatum)* were amplified by using the Universal Primers ITS1-ITS4.

The isolates of *T. asperellum* showed a resemblance with other isolates of the same species belonging to neighbouring countries like India and China with 98% bootstrap (BS) value/1000 replicates. All the isolates were categorized under the same clade representing their existence and similarity with *T. asperellum.* The fungus *Alternaria alternata* was used as an outgroup to determine the distance between the clades (Figure 4). The existence of *T. viride* isolates in the main clade with 100% BS value has supported their presence with the isolates of India, Egypt and Germany. The collected isolates from Pakistan were further categorized under the subclade with 96% BS value/1000 replicates representing the diminutive assortment (Figure 5).

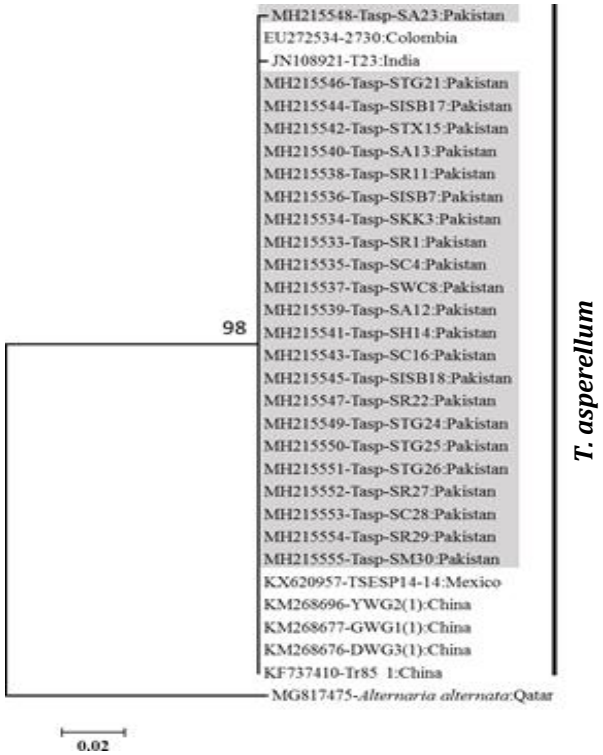

Figure 4. The phylogenetic analysis of *T. asperellum* was conducted with previously recorded GenBank sequences with the MP method. The proportion of trees in which the related taxa are grouped is revealed next to the branches. The analysis intricate 31 base sequences. All the positions comprising gaps and mislaid data were removed.

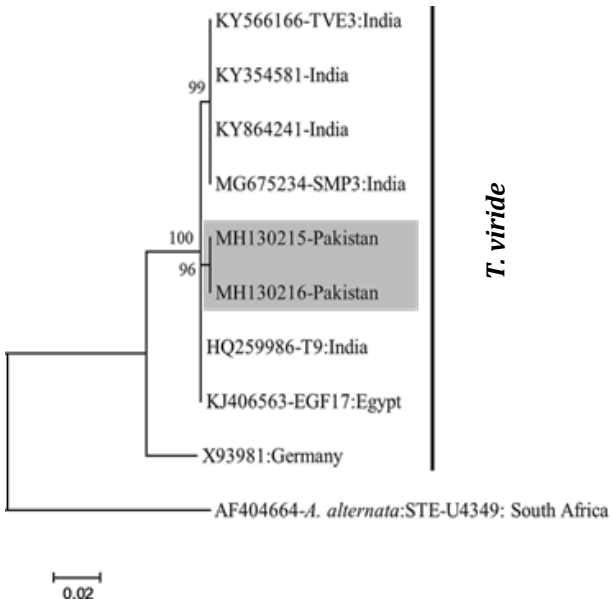

Figure 5. The phylogenetic analysis of *T. viride* was conducted with previously recorded GenBank sequences with the MP method. The proportion of trees in which the related taxa are grouped is revealed next to the branches. The analysis intricate 10 base sequences. All the positions comprising gaps and mislaid data were removed.

The phylogenetic analysis of various isolates of *T. harzianum* showed their presence in the subclade under the main clade with 96% BS value. The other isolates of India, China, Iran and Poland were categorized under the subclades with 62, 71, 94 and 94% bootstrap values (Figure 6). The collected isolate of *T. hamatum* resembled the isolates of *T. hamatum* reported from the other countries with 99% BS value/1000 replicates. All the isolates of *T. hamatum* isolated from different countries existed under the same main clade showing their uniformity with the Pakistani isolate (Figure 7). The collected isolates of *T. longibrachiatum* showed their presence with the identical isolates reported from different continents like India, China, Korea, Spain etc. with 97% BS value/1000 replicates. A single subclade was also found in the main subclade representing the variation in already reported isolates from different countries. The intraspecies analysis of *Trichoderma* isolates with the maximum parsimony method (MP) showed the resemblance of collected isolates with their identical isolates.

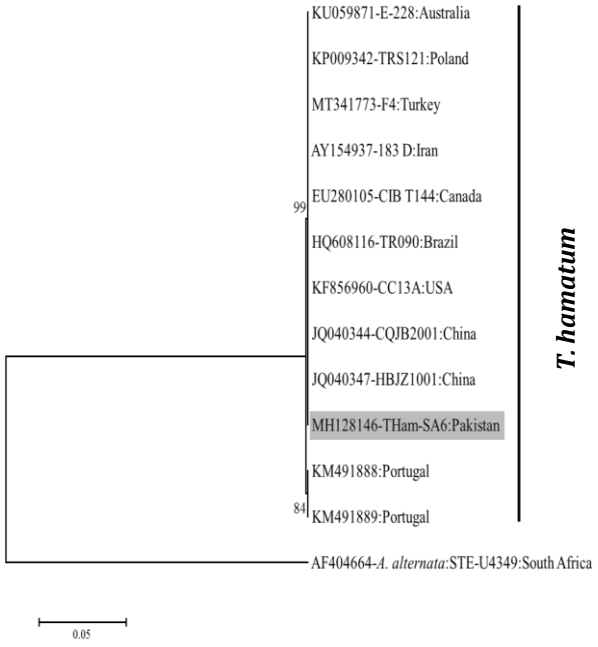

Figure 6. The phylogenetic analysis of *T. hamatum* was conducted with previously recorded GenBank sequences with the MPmethod. The proportion of trees in which the related taxa are grouped is revealed next to the branches. The analysis intricate 13 base sequences. All the positions comprising gaps and mislaid data were removed.

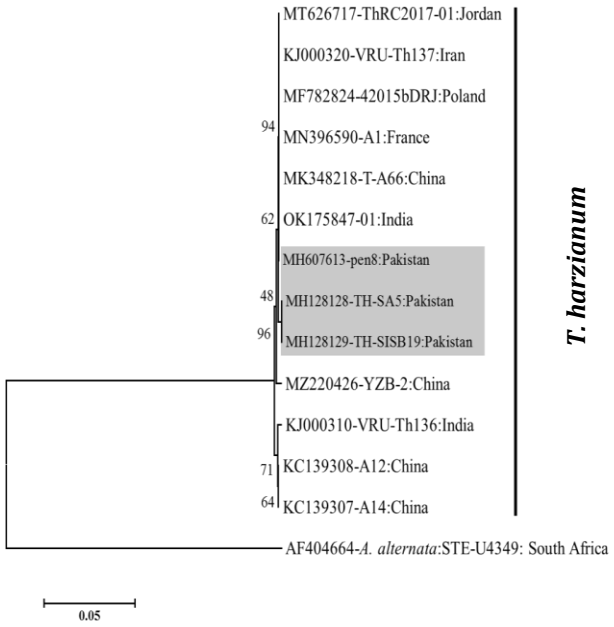

Figure 7. The phylogenetic analysis of *T. harzianum* was conducted with previously recorded GenBank sequences with the MP method. The proportion of trees in which the related taxa are grouped is revealed next to the branches. The analysis intricate 14 base sequences. All the positions comprising gaps and mislaid data were removed.

The isolates of *T. asperellum* existed under the second subclade with 95% BS value/1000 replicates and were closely related to the isolates of neighbouring countries. *T. hamatum* resembled the isolates reported from USA, Portugal and China with 87% BS value existing under the main clade with 100% BS value/1000 replicates. The phylogenetic analysis of *T. viride* showed their resemblance to similar species with a 92% BS value. The identified isolates were linked to the reported isolates of neighbouring countries and existed in the subclade representing little variation (Oskiera *et al.*, 2015). Similarly, the evolutionary relationship of *T. longibrachiatum* has placed the collected isolates in similar clades comprising identical species with a 69% BS value. The collected isolates of *T. harzianum* were categorized under the subclade due to a diversified population with a 96% BS value. The main clade contains the similar *Trichoderma* spp. that belongs to the adjacent countries with 98% BS value based on 1000 replicates (Figure 8).

The phylogenetic relationship constructed between biocontrol (*Trichoderma viride*) and pathogenic

(*Trichoderma viride*) isolates revealed that beneficial strains clustered in a separate clade as per their similarity forming a sister clade with the pathogenic isolates of the same species. *T. harzianum* beneficial strains from Iran and China form well-defined separate clades compared to the pathogenic isolates F152 and F153 as shown in the red box (Figure 10) which form sister clade with each other and within the same species subclade. Biocontrol isolates of *Trichoderma* were in different clades whereas pathogenic strains of *Trichoderma viride* were in separate clades as described in Figure 10. In the case of *Trichoderma* spp., a variation in the ITS section allowed *T. viride* and *T. harzianum* to be identified.

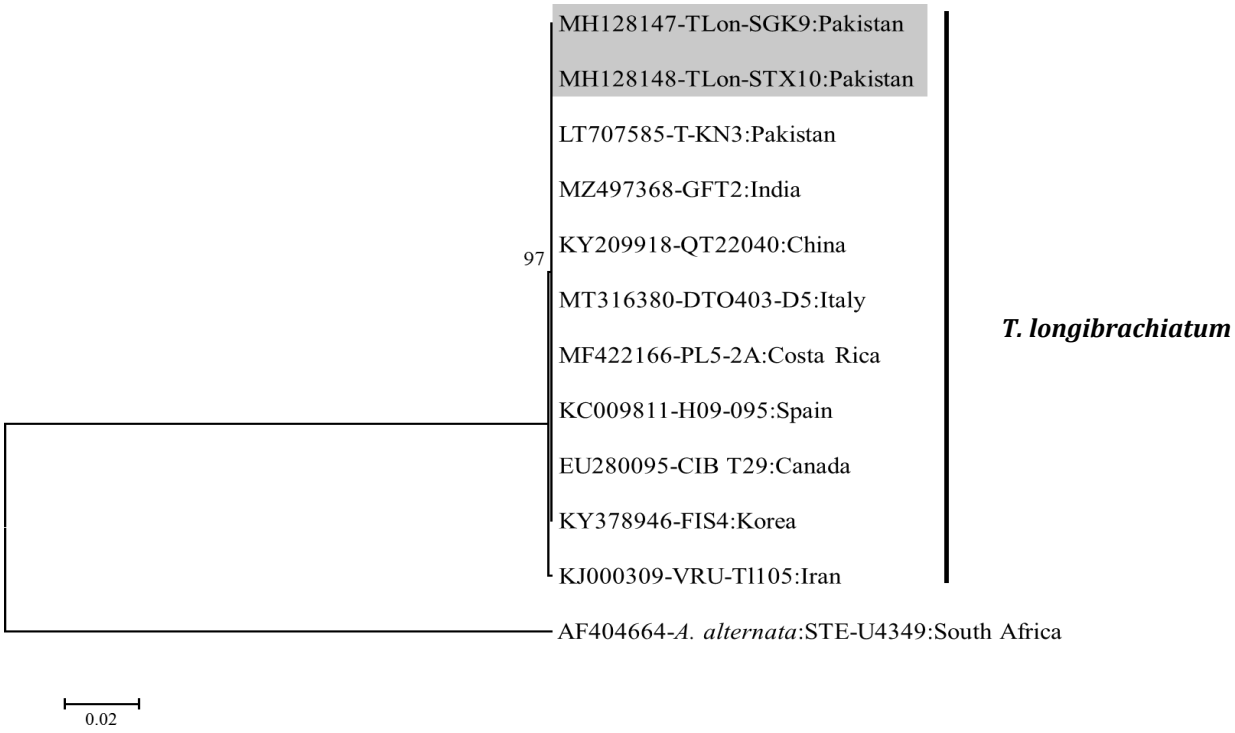

Figure 8. The phylogenetic analysis of *T. longibrachiatum* was conducted with previously recorded GenBank sequences with the MP method. The proportion of trees in which the related taxa are grouped is revealed next to the branches. The analysis intricates 12 base sequences. All the positions comprising gaps and mislaid data were removed.

DISCUSSION

Plant diseases are prone to biotic and abiotic factors resulting in heavy yield reduction in crops. The diseases controlled by extensive usage of synthetic chemical pesticides resulting unwanted health, safety and environmental menace (Sarwar, 2015). The infective effect of plant pathogens has to be minimized by the applications of biological control agents (BCA) which is the safest and long-lasting method of pathogen(s) control. Many opportunistic virulent plant symbionts have also been categorized under this genus which is a serious effect of concern (Harman *et al.*, 2004). *Trichoderma* was easily isolated from soil and root by conventional methods, primarily due to its rapid growth, abundant conidiation, and colonization of organic substrates, which facilitate rapid development on various substrates (Gupta *et al.*, 2014). The results obtained in this study agreed with those reported by different authors showing that *Trichoderma* spp. is a fungus found in many types of environments around the world (Rivera-Méndez *et al.*, 2018).

The taxonomy of *Trichoderma* isolates has been characterized based on the variations in its structural features and sporulation. The *Trichoderma* species employs several mechanisms to control the development and spread of injurious pathogens such as parasitism, competition and antibiosis. A biological control agent such as *Trichoderma* is an essential part of integrated pest management (IPM) for pest and disease control in an environmentally responsible way (Heydari and Pessarakli, 2010).

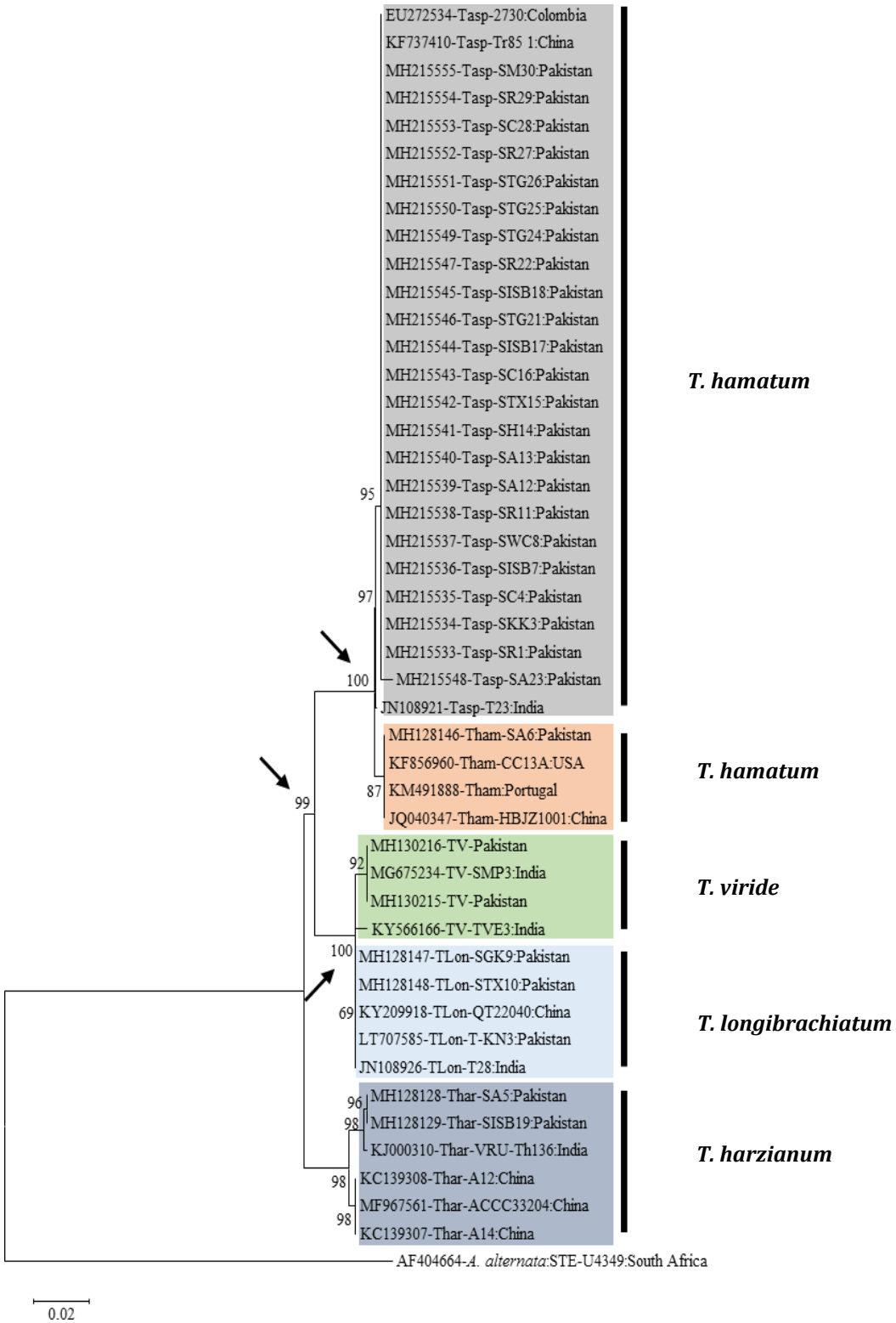

Figure 9. The combined phylogenetic analysis of *T. harzianum, T. hamatum, T. longibrachiatum, T. asperellum* and *T. viride* was conducted with previously recorded GenBank sequences with the MP method. The proportion of trees in which the related taxa are grouped is revealed next to the branches. The analysisintricates 46 base sequences. All the positions comprising gaps and mislaid data were removed. The fungus *A. alternata* was used as an outgroup to determine the distance between the clades.

The current study was designed to evaluate the genetic diversity of the isolated *Trichoderma* species based on their morphological and molecular attributes. *Trichoderma* spp., have been varied based ontheir colony colour, growth, mycelium, odour, conidia, conidiophore and phialides and categorized under different morphological sections (Shah *et al.*, 2012). The characteristics of the fungal organism observed in the micro and macroscopic studies are consistent with the genus of *Trichoderma* sp. Several scientists also observed the similar characteristics (Andrade-Hoyos *et al.*, 2019; Chaverri *et al.*, 2015; du Plessis *et al.*, 2018; Jang *et al.*, 2018; Nawaz *et al.*, 2018). Variations between the *Trichoderma* isolates may be influenced by changes in geographical area and soil type. The morphological identification of the fungal pathogens is the preliminary and reliable criterion to study the invasive species in detail. Different *Trichoderma* spp. isolated from soil samples were categorized and identified based on their taxonomic features like colony morphology and spore structures (Sharma and Singh, 2014). Morphological identification of *Trichoderma* isolates has been considered to be the most potent and basic criterion to diagnose the isolates at the genus level (Błaszczyk *et al.*, 2011). The scientists worked on the characterization of *Trichoderma* spp. and distributed the isolates under different groups based on the branching pattern of conidiophores, short extravagant phialides with the plane and small spores (conidia) (Kumar *et al.*, 2012). *T. asperellum* has conidiophores terminating 2 or more phialides and primary branching arising at nearly 90 degrees to the main axis. *T. longibrachiatum*, the conidiophores were complicated and progressively longer, often paired, secondary branched. Phialides arise directly from secondary branches, typically not in whorls. Earlier studies revealed the presence of *T. harzianum. T. hamatum* and *T. viride* in these regions. Different researchers reported that the morphological characteristics were commonly fluctuating, making them deficient in species identification. Variations were observed among *Trichoderma* spp. according to the dimensions or diameters of chlamydospores and conidia. On malt extract agar (MEA), the isolates produced larger colonies, Czapek dox agar did not contain aerial mycelium mates, while yellow pigmentation was observed in some MEA isolates. Temperature fluctuation has also been observed with *T. harzianum*, which has evolved into different patterns on various nutrient mediums (Küçük and Kivanç, 2004).

The phylogenetic species concept based on a concordance of multiple gene genealogies has revolutionized fungal taxonomy (Taylor *et al.*, 2000) and exposed weaknesses in traditional morphology-based identification. Morphologically identified *Trichoderma* spp., were precisely identified and confirmed by using the Internal transcribed spacer (ITS) region that led to a product of 600 bp in all the isolates. ITS is a conserved rDNA sequence that has been widely used to characterize and perform the phylogenetic analysis of fungal isolates (Skouboe *et al.*, 1999). Different researchers evaluated the twelve *Trichoderma* spp. based on their morpho-molecular features that have been collected in the rhizosphere area of tomato plants in Saudi Arabia. The ITS region was used inthe detailed species-wide identification of *Trichoderma* isolates (Mazrou *et al.*, 2020). The genomic analysis of 69 *Trichoderma* spp. biocontrol agents revealed that numerous isolates were incorrectly classified as *T. viride* (Hermosa *et al.*, 2004). Three biocontrol agents previously identified as *T. viride* (ATCC36042, ATCC52439, and ATCC52442) have now been reclassified as *T. asperelloides* (Samuels *et al.*, 2002).

As technology is advancing day by day, DNA-based methods have been routinely used to accurately identify the species, for example, the *Trichoderma* strains were morphologically identified as *T. harzianum* but molecular identification suggests that they should be identified as *T. hamatum*, *T. longibrachiatum*, and *T. atroviride* (Hermosa *et al.*, 2004). Furthermore, molecular identification approaches identified new strains from *Trichoderma* species that were not yet taxonomically established (Oskiera *et al.*, 2015).

T. viride was detected in just two (BBA70239 and GJS89-142) of the 69 biocontrol agents reported by Hermosa *et al.* (2004). None of these isolates has been tested as a biocontrol agent, according to available information (Chaverri *et al.*, 2015). This could indicate that the pathogenic behaviour of *T. viride* is restricted to a certain molecular clade. Previous investigations exhibited that how the *T. viride* strain infecting *Pinus nigra* seedlings differs from the *T. viride* biocontrol strain. The harmful behaviour of *T. viride* appears to be at odds with other accounts portraying *Trichoderma* species as avirulent plant symbionts or parasites of other fungi (Harman *et al.*, 2010).

The molecular findings provide the comprehensive and

reliable conclusions that will be adopted for determining potentially beneficial/pathogenic strains of *Trichoderma*. Thus an integrated approach of morphological and molecular markers can be employed to identify a superior strain of *Trichoderma* for its commercial exploitation and open several gateways for future research in the field of applied biotechnology.

CONCLUSION

Trichoderma has been widely distributed in rhizospheric soils of various vegetable hosts in the Punjab province. A systematic survey showed that *Trichoderma* populations associated with soils of different vegetable hosts in different districts of Punjab, Pakistan exhibited a clear picture of the distribution of species in the region. The cultured *Trichoderma* isolates were morphologically distinct and the same were confirmed by molecular analysis. These findings suggests that the species identified were: *T. harzianum, T. hamatum, T. longibrachiatum, T. asperellum* and *T. viride* with a sequence identity of 98% to 100%. Sequence analysis revealed that the present study *Trichoderma* isolates showed maximum homology with isolates earlier reported from India, China, Egypt, Germany, USA, Portugal, Korea and Spain. Several studies have reported the biocontrol potential of *Trichoderma* isolates. These finding will be helpful for students and researchers to find out the effective biocontrol agent against native phytopathogens followed by the commercial production of superior strain of *Trichoderma*.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authors contributed equally to this work.

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